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13. Abstract ***(Maximum 200 Words)*** ***(abstract should contain no proprietary or confidential information)***

Studies of the genetics of populations that were historically endogamous for reasons of geography, religion, or language have contributed substantially to our understanding about hereditary predisposition to cancer. One such population is found in the Netherlands where multiple, prevalent, population-specific founder mutations have been identified. Genetic susceptibility to prostate cancer is being investigated in the Netherlands Cohorts Study (NLCS) on Diet and Cancer, which has identified over 800 cases of prostate cancer among 58,279 male participants. From this study, we have identified 300 cases and 300 controls from men of comparable age to identify markers near prostate cancer susceptibility genes that are present at higher frequency in the group of men with prostate cancer. We optimized the methods for analysis of these markers and observed a candidate region of association on chromosome 1.

14. SUBJECT TERMS
Prostate cancer, linkage disequilibrium, association

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INTRODUCTION

This study uses several observations about the genetic basis of prostate cancer to enhance the efficiency of identifying susceptibility genes. 1) Prostate cancer is a multi-step genetic disorder in which some of the observed genetic alterations in prostate cancer cells were acquired through the germline. 2) The chromosomal locations of some of these genes can be identified readily in prostate cancer cells on the basis of their demonstrating loss of heterozygosity. 3) Historically, certain populations have been endogamous causing them to have more genetic homogeneity and to have prevalent founder mutations in some of their disease susceptibility genes. As a result of the population's endogamy, short chromosomal regions have remained identical by descent, leading to recognizable associations of the founder mutations with linked marker alleles (*linkage disequilibrium*). The Dutch represent such a population.

BODY

The project started six months late in the Netherlands because of delays in contract negotiation. An additional 6-month delay occurred as the result of the terrorist events in New York City of 11 September 2001, the recruitment of qualified technical personnel to perform the work both in New York and in the Netherlands (previously noted in a letter for a no-cost extension) and from receiving samples from Dutch pathology laboratories. A timetable for the statement of work and the accomplishments for each of the tasks follows.

Task 1A. Subject identification and selection. Months 6-20

The medical histories of each subject were reviewed, confirming diagnosis of prostate cancer, and noting age and TMN classification at time of diagnosis. Tissue blocks were obtained from non-cancerous tissues (usually lymph nodes) and thick (50 micron) sections were cut. DNA was purified from these sections using a protocol optimized in our laboratory and then quantified. To extend the utility of these sections, a technique for whole genome amplification using primer extension preamplification (PEP) was optimized to provide 50-fold amplification, then applied to the samples.

At the time that the proposal was submitted, we postulated that 800 cases would be available at the time that we initiated this study. This is based on the projection that 8.3 years of follow-up information. In fact, 7.3 years of follow-up information was available and 704 (not 641) cases were identified. From these, we collected samples for 498 cases from the Dutch pathology laboratories. We then selected the 300 samples from the normal tissues. This met our goal of generating 48,000 genotypes from *cases* - 160 genotypes per *case*. The remainder of the 96,000 genotypes budgeted in the proposal came from controls (See below.)

Task 1B. Control identification and selection. Months 6-20

Buccal swab samples were collected from the whole subcohort of the Netherlands Cohort Study on Diet and Cancer and DNA was extracted from these samples. This included buccal swabs from 940 males. From these, 300 controls with the highest yields of DNA were selected for PEP amplification and genotyping. These samples have been found to be representative of

the whole NLCS. We then selected the 300 samples from the normal tissues. This met our goal of generating 48,000 genotypes from *controls* - 160 genotypes per *control*, or the remainder of the 96,000 genotypes budgeted in the proposal.

Task 2A. Markers from regions associated with loss of heterozygosity in prostate cancer not currently available in the laboratory will be identified and fluorochrome-labeled primers will be synthesized. Months 1-12

We identified microsatellite markers for each of the following chromosomal regions 1q24-q25, 7q31, 8p21-p22, 10q23-q25, 13q14, 16q22, 17p, 17q21-q22, Xq11-q13. Because of uncertainties about relative map positions, we confined our markers to those which have shown (LOH) in a high proportion of subjects in a single report, to those which show (LOH) in more than one report, or to those whose map positions were known with a high degree of confidence from the GeneMap99 (http://www.ncbi.nlm.nih.gov/GeneMap99) and which were tightly linked to markers that show LOH. In addition, we added markers for the following chromosomal regions that have shown linkage to prostate cancer susceptibility in families with multiple affected members, 1q24-25, 1q42-43, and Xq27-28 (Smith, et al., 1996, Cooney, et al., 1996, Gronberg, et al., 1997, Xu, et al., 1998, Berthon, et al., 1998) Recently, we remapped some of these markers using the annotated human genome map (http://genome.ucsc.edu) and discovered that the average spacing of our markers was 2 Mb, i.e. at the density originally planned (table 1). However, some of the markers did not map to the chromosomes to which they were originally assigned. These were excluded from subsequent analysis.

Task 2B. Standard PCR conditions will be developed for each of these markers. Months 1-12

The primer sequences for each of these markers was identified using standard databases (http://www.gdb.org). The predicted sizes of the PCR product alleles were noted and markers yielding products of different predicted sizes were grouped and labeled with one of three different fluorescent dyes (tet, fam, hex). The net effect of this grouping is that multiple markers can either be amplified simultaneously and/or pooled from separate amplifications to minimize the number of electrophoretic runs. Procedures for pooling separate amplification reactions have been optimized. Different thermostable enzymes were tested for their fidelity for amplifying microsatellites, including AmpliTaq, AmpliTaq Gold, Platinum Taq, Platinum Tsp, and Expand High Fidelity. Among these enzymes, Platinum Tsp (Life Technologies, Gaithersburg, MD) was found to produce the most reliable amplification with the least stutter and the least random addition of an adenine at the 3' end of the PCR product. For each of the markers, different PCR conditions were tested, varying temperature and magnesium chloride concentrations, and the optimum conditions were defined.

Task 2C. Individuals with alleles of known sizes will be identified for use in subsequent genotyping analyses. Months 1-12

Individuals with alleles of known sizes were identified for use in subsequent genotyping analyses.

Table 1: Revised Microsatellite Positions Based on Human Genome Annotation

Marker	Chr	Position	Segmental difference	Marker	Chr	Position	Segmental difference
D1S243	1	2.01	3.99	D8S264	8	2.12	1.55
D1S2870	1	6.00	0.67	D8S262	8	3.66	2.54
D1S214	1	6.67	0.38	D8S1742	8	6.20	0.30
D1S2694	1	7.05	4.15	D8S277	8	6.50	2.31
D1S2667	1	11.20	2.15	D8S351	8	8.81	0.46
D1S228	1	13.35	0.77	D8S503	8	9.27	1.32
D1S407	1	14.12	0.27	D8S520	8	10.59	0.72
D1S507	1	14.39	4.07	D8S265	8	11.32	1.44
D1S2644	1	18.46	0.97	D8S552	8	12.75	0.09
D1S199	1	19.43	0.55	D8S1106	8	12.85	1.84
D1S2843	1	19.98	1.09	D8S511	8	14.69	0.14
D1S478	1	21.07		D8S1827	8	14.83	0.42
D1S2844	1	160.14	5.14	D8S1731	8	15.25	0.41
D1S2799	1	165.28	2.43	D8S549	8	15.66	0.96
D1S452	1	167.71	1.24	D8S254	8	16.62	3.22
D1S2815	1	168.95	2.79	STS-M15856	8	19.83	0.54
D1S218	1	171.74	3.58	D8S258	8	20.38	1.05
D1S212	1	175.32	1.55	D8S282	8	21.43	0.19
D1S215	1	176.87	0.48	D8S560	8	21.61	0.84
D1S2883	1	177.34	0.56	D8S136	8	22.46	0.00
D1S117	1	177.90	1.64	D8S1786	8	22.46	0.23
D1S466	1	179.54	2.43	D8S1752	8	22.69	0.13
D1S2127	1	181.98	2.81	D8S1734	8	22.82	2.02
D1S518	1	184.79	0.22	SHGC-6135	8	24.84	5.58
D1S222	1	185.01	0.38	D8S339	8	30.41	3.27
D1S238	1	185.39	3.28	D8S283	8	33.69	1.67
D1S422	1	188.67	3.36	D8S87	8	35.36	0.11
D1S2757	1	192.03	3.88	D8S1750	8	35.47	2.25
D1S413	1	195.91		D8S1722	8	37.72	2.18
D1S213	1	?		D8S255	8	39.90	1.36
D1S2827	1	213.20	1.01	D8S268	8	41.26	10.93
D1S490	1	214.21	2.81	D8S587	8	52.20	
D1S2758	1	217.01	2.20	D8S1130	?		
D1S2871	1	219.21	9.54	D8S133	?		
D1S251	1	228.75	0.32	D8S201	?		
D1S2709	1	229.07	2.70	D8S261	?		
D1S446	1	231.76	1.17	D8S298	?		
D1S235	1	232.93	0.74				
D1S2850	1	233.67	3.99				
D1S180	1	237.66	0.26				
D1S2785	1	237.92	1.90				
D1S2842	1	239.82	0.82				
D1S2811	1	240.64	3.18				
D1S2836	1	243.82					

Marker	Chr	Position	Segmental difference	Marker	Chr	Position	Segmental difference
D10S195	10	76.96	0.94	D17S938	17	6.45	0.00
D10S202	10	77.91	2.29	D17S796	17	6.45	1.01
D10S219	10	80.20	5.24	D17S960	17	7.46	1.55
D10S1658	10	85.44	4.22	D17S786	17	9.01	28.51
D10S541	10	89.66	0.82	D17S250	17	37.53	1.90
D10S1739	10	90.48	3.56	D17S800	17	39.43	1.15
D10S583	10	94.03	0.82	D17S776	17	40.58	1.00
D10S185	10	94.85	1.95	D17S855	17	41.58	0.03
D10S571	10	96.80	2.35	D17S1323	17	41.61	0.24
D10S1709	10	99.15	1.60	D17S1327	17	41.85	3.48
D10S198	10	100.74	1.36	D17S791	17	45.33	
D10S192	10	102.10	1.94	D17S1810	?		
D10S1697	10	104.04	0.95	D17S856	?		
D10S222	10	104.98	1.53	DXS1047	X	127.78	8.23
D10S1671	10	106.52	4.38	DXS1062	X	136.01	1.07
D10S597	10	110.90	2.09	DXS1192	X	137.07	0.91
D10S1682	10	112.99	3.32	DXS1232	X	137.99	0.21
D10S562	10	116.30		DXS1205	X	138.20	1.30
D10S205	12	48.06		DXS1227	X	139.50	1.38
D10S532	?			DXS8106	X	140.88	1.82
D10S1644	?			DXS8043	X	142.70	0.21
D16S398	16	65.91	7.94	DXS8028	X	142.91	1.50
D16S512	16	73.85	2.45	DXS1200	X	144.41	1.06
D16S515	16	76.30	1.62	DXS548	X	145.47	0.84
D16S518	16	77.92	0.78	DXS6687	X	146.31	0.78
D16S3049	16	78.70	0.12	DXS1193	X	147.09	0.55
D16S3096	16	78.82	0.08	DXS8011	X	147.64	3.00
D16S516	16	78.90	0.04	DXS8061	X	150.64	2.65
D16S504	16	78.95	0.49	DXS1108	X	153.29	0.22
D16S3040	16	79.43	0.42	DXS1107	X	153.51	
D16S507	16	79.86	2.84	DXS8103	?		
D16S422	16	82.69	3.61	DXS1177	?		
D16S520	16	86.30	0.60	DXS1113	?		
D16S476	16	86.89	0.78				
D16S413	16	87.68					
D16S402	?						
D16S289	?						
D16S347	?						

Task 2D. none indicated in original proposal.

Task 3A. Genotype analysis of each marker will be performed for each individual. Months 36-46

Genotyping for each individual in the study was performed for each of the markers. Allele calling was performed using the ABI genotyper software. Whenever possible, genotypes for multiple markers, each tagged with a different fluorochrome were run in the same lane to conserve on the number of electrophoretic runs. Each lane contained internal controls for calibration. Samples with alleles of known size were included in each run to validate the calibration. In addition, one or more samples were typed in duplicate in each run to assess the reproducibility of results.

Task 3B. Statistical analysis will be performed. Months 36-46

Standard calculations of odds ratios using contingency tables are performed for cases and controls to estimate the risks associated with each marker (table 2). Allele frequencies were identified to determine where the risks might be spurious based on small sample sizes (Figure 1).

Task 4A. To assure the quality of the data, a sample will be reanalyzed and repeat genotyping were performed, if indicated.

Each of the microsatellites was scored independently by at least two individuals and entered into a spreadsheet. When the results were discordant, then they met to resolve these discordances. When that was not possible, then the analyses were repeated.

Task 4B. A final report has been prepared.

The most interesting results were observed for chromosome 1 markers suggesting the possibility of one or more susceptibility loci on that chromosome. Significant associations were observed at D1S466/allele 163 (OR 1.74, 95% CI: 1.16-2.62). A similar association was observed for the linked marker, D1S2127/allele 122 (OR 1.58, 95% CI: 1.06-2.36). Other associations were seen for D1S218/ allele 284 (OR 2.03, 95%CI: 1.22-3.38) and D1S466/allele 155 (OR 6.73, 95%CI: 1.50-30.08), but these observations were based on small numbers of allele.

To test whether RNASEL, the candidate tumor suppressor gene on this region of chromosome 1, might be involved in prostate cancer pathogenesis through a 2-hit mechanism (Rokman, et al., 2002), immunocytochemistry was performed on 100 prostate cancer samples in a tissue microarray using an RNASEL-specific antibody. No differences in staining were observed between the normal prostate and the tumor, thus disproving this hypothesis.

KEY RESEARCH ACCOMPLISHMENTS

Development of DNA databases from cases and controls for genomic analysis.

Completed collection, DNA extraction, and selection of samples for this study.

Development of high-quality, reproducible methods for microsatellite typing

Development of high-quality, reproducible methods for whole genome amplification

Identification of a candidate region for prostate cancer pathogenesis that can be resolved using higher resolution association and LOH studies.

REPORTABLE OUTCOMES

Proposal, "Mentorship Program in Prostate Cancer Genetics" K24 (CA85326-01A1), was funded by the National Cancer Institute.

Rene Vogels Award, Dutch Society of Oncology to Maurice Zeegers, Ph.D., co-investigator, for research on genetic susceptibility to prostate cancer.

Table 2: Odds Ratios for Chromosome 1 Markers

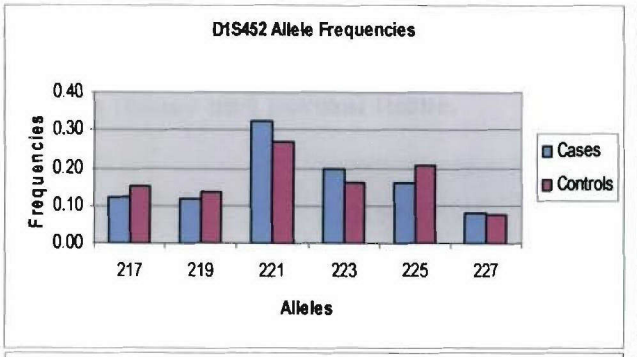
D1S452			
Allele	**Odds ratio**	**lower 95% confidence interval**	**higher 95% confidence interval**
217	0.77	0.48	1.23
219	0.83	0.51	1.34
221	1.31	0.92	1.87
223	1.29	0.85	1.97
225	0.73	0.48	1.11
227	1.07	0.58	1.97
D1S2815			
210	1.00	0.39	2.56
212	0.64	0.29	1.38
214	0.63	0.36	1.12
216	0.91	0.56	1.50
218	1.03	0.68	1.55
220	1.21	0.77	1.91
222	1.35	0.87	2.08
224	0.65	0.35	1.20
226	1.12	0.58	2.17
228	1.08	0.50	2.35
230	1.26	0.49	3.25
232	2.01	0.18	22.33
234	-	-	-
236	-	-	-
D1S218			
266	0.25	0.05	1.18
268	0.53	0.22	1.26
270	0.42	0.19	0.92
272	0.29	0.15	0.55
274	0.75	0.46	1.21
276	0.98	0.62	1.56
278	1.12	0.68	1.84
280	1.43	0.91	2.25
282	1.48	0.95	2.30
284	2.03	1.22	3.38
D1S2883			
179	1.68	0.60	4.70
181	0.85	0.39	1.87
183	1.43	0.84	2.44
185	1.27	0.85	1.88
187	1.04	0.70	1.55
189	0.79	0.51	1.23
191	0.52	0.25	1.08

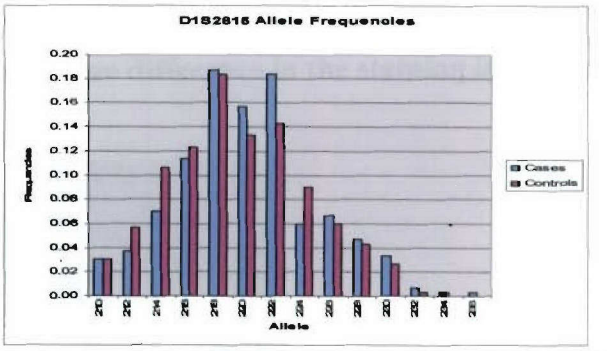
193	0.67	0.32	1.38
195	1.05	0.56	1.95
197	0.94	0.48	1.83
D1S117			
120	0.80	0.48	1.33
122	1.28	0.77	2.15
124	0.68	0.44	1.04
126	0.75	0.51	1.11
128	1.34	0.91	1.97
130	1.47	0.94	2.29
D1S466			
155	6.73	1.50	30.08
157	1.53	0.76	3.07
159	1.32	0.76	2.31
161	0.73	0.44	1.23
163	1.74	1.16	2.62
165	1.06	0.66	1.69
167	0.75	0.47	1.20
169	0.65	0.36	1.16
171	0.68	0.35	1.31
173	0.55	0.27	1.14
175	-	-	-
D1S2127			
112	1.51	0.25	9.07
114	1.16	0.54	2.49
116	0.39	0.19	0.78
118	0.44	0.25	0.76
120	0.79	0.50	1.24
122	1.58	1.06	2.36
124	1.39	0.91	2.12
126	1.39	0.86	2.25
128	1.34	0.76	2.36
130	0.24	0.05	1.16
132	0.50	0.09	2.73
D1S518			
191	1.50	0.25	9.04
193	0.83	0.49	1.41
195	1.53	0.88	2.68
197	1.57	0.93	2.65
199	1.16	0.72	1.87
201	0.78	0.46	1.31
203	1.00	0.58	1.71
205	1.00	0.57	1.73
207	1.20	0.70	2.06
209	0.94	0.48	1.83

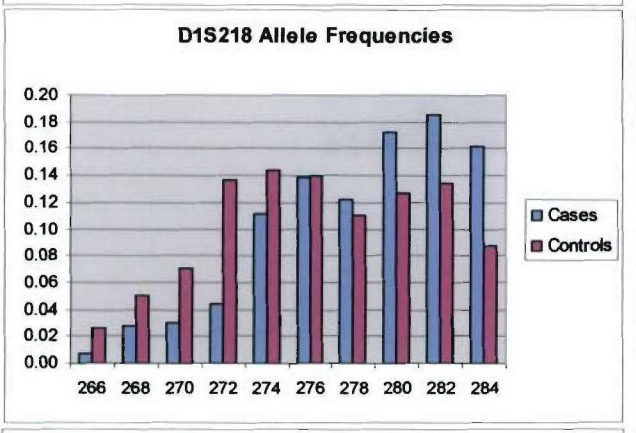
211	0.66	0.23	1.87
213	0.56	0.16	1.95
215	0.49	0.12	1.99
217	0.20	0.02	1.69
219	0.39	0.08	2.05
221	0.00	-	-
223	0.00	-	-
D1S222			
258	0.00	-	-
260	0.16	0.04	0.74
262	0.68	0.35	1.34
264	0.96	0.50	1.88
266	1.13	0.61	2.09
268	0.90	0.56	1.47
270	0.81	0.52	1.24
272	1.26	0.85	1.86
274	1.42	0.94	2.14
276	1.13	0.61	2.09
D1S422			
157	0.87	0.54	1.39
159	0.82	0.54	1.24
161	0.95	0.64	1.40
163	1.08	0.75	1.56
165	1.14	0.73	1.76
167	1.54	0.77	3.10

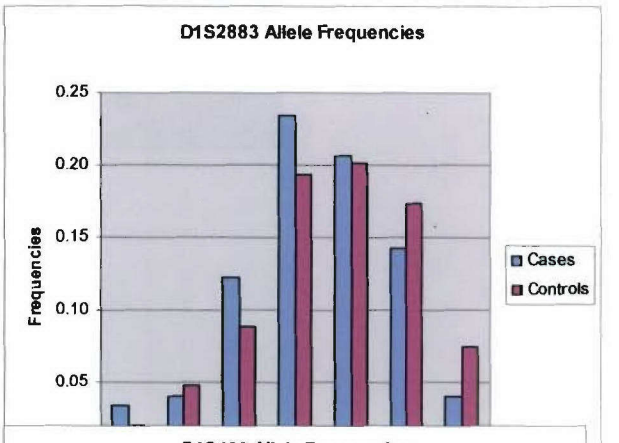
Figure 1: Allele Frequencies of Chromosome 1 Markers (next page)

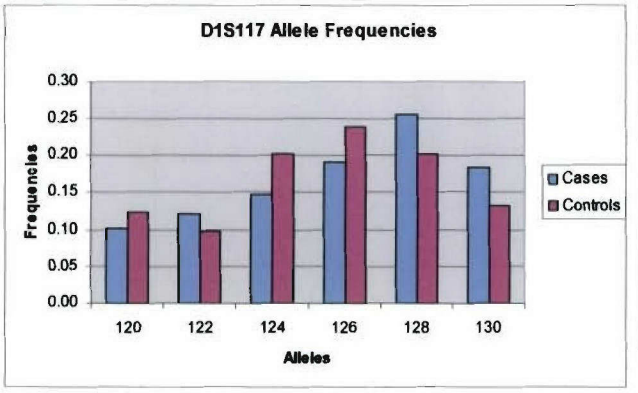
Allele frequencies for each of the chromosome 1 markers were analyzed. Significant differences in allele frequencies between cases and controls are highlighted by arrows.


D1S452 Allele Frequencies
Frequencies
0.40
0.30
0.20
0.10
0.00
217
219
221
223
225
227
Alleles
Cases
Controls


D1S2815 Allele Frequencies
Cases
Controls


D1S218 Allele Frequencies
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
266
268
270
272
274
276
278
280
282
284
Cases
Controls


D1S2883 Allele Frequencies
Frequencies
0.25
0.20
0.15
0.10
0.05
Cases
Controls


D1S117 Allele Frequencies
Frequencies
0.30
0.25
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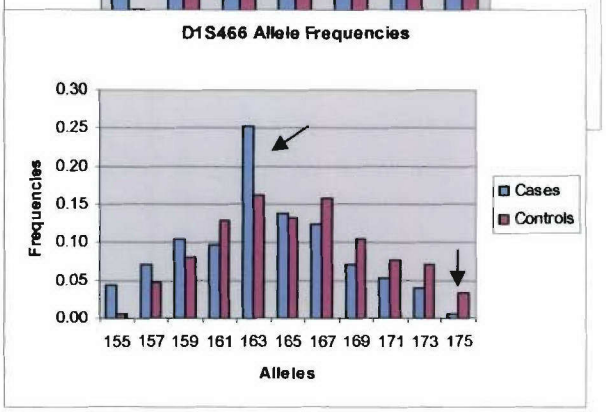
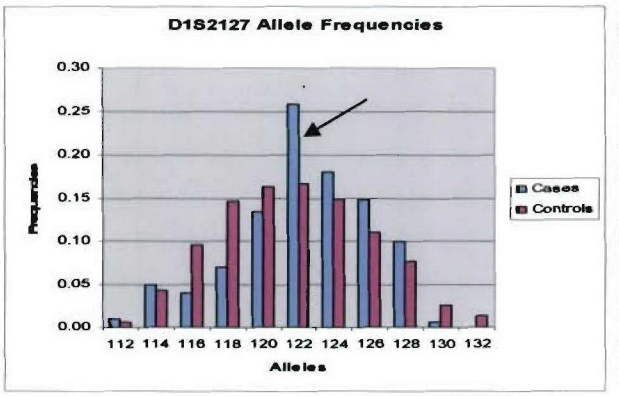
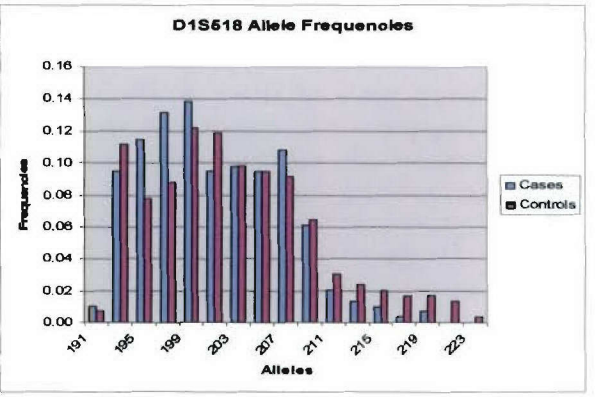
0.15
0.10
0.05
0.00
120
122
124
126
128
130
Alleles
Cases
Controls


D1S466 Allele Frequencies
Frequencies
0.30
0.25
0.20
0.15
0.10
0.05
0.00
155 157 159 161 163 165 167 169 171 173 175
Alleles
Cases
Controls


D1S2127 Allele Frequencies
Frequencies
0.30
0.25
0.20
0.15
0.10
0.05
0.00
112 114 116 118 120 122 124 126 128 130 132
Alleles
Cases
Controls


D1S518 Allele Frequencies
Frequencies
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
191
195
199
203
207
211
215
219
223
Alleles
Cases
Controls

CONCLUSIONS

This work identified a candidate region on chromosome 1 for prostate cancer pathogenesis. This works demonstrates the feasibility for high-throughput multiplex microsatellite marker analysis and the feasibility for extending small samples of DNA 50-fold for genetic analysis. This creates the foundations for other case-subcohort studies

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